May 3
Our first day in the Lab. We started off by taking an inventory of reagents and supplies to figure out what we had and what we needed to order. Organization and inventory of the fridges and freezes also started but the -20°C freezer needed to thaw after having sat for two years. A number of items were sent down to autoclave to ensure that we have materials ready for work. Lastly, agar plates with antibiotics were made following the protocol for LB Media.

May 4
We continued to work on the organizing the -20 once it thawed. We also started to tackle the fridge. There were several dozen plates that had been sitting in the fridge, drying out, over the last two years. The biggest problem was dealing with poorly labelled tubes or tubes that had no label. When in doubt, throw it out!

After looking through the -80 freezer, Dan found some *E. coli* cells (3073C and TOP10) and we used these to test our plates. There were both competent *E. coli* and *E. coli* with chloramphenicol (Chlor) resistance. Cells were spread onto each type of plate (Chlor and Amp) and into liquid media (Chlor)

May 5
Unfortunately, the Chlor resistant *E. coli* were able to grow on the Amp plates, there was a lawn of red cells. Indicating that the concentration of ampicillin (Amp.) was not great enough. There may have been some contamination of the Amp plates, as odd yellow growths were observed.

Organization of the freezer continued. There was a lot in there…

Overnight cultures were made of TOP10 cells and C3037 cells. Two 5mL cultures were made of each bacteria. They were incubated at 37°C and 200rpm

Solutions for making cells competent were made: 50mM $CaCl_2$ (5mL 1M $CaCl_c$ and 95mL of ddH_2O) & 50mM $CaCl_2$ with 15% glycerol (5mL 1M $CaCl_2$, 15mL Glycerol, 80mL ddH_2O)

May 6
Made competent cells following the competent cell protocol. Four subcultures were made, two for each of the TOP10 and C3037. It took approximately four hours to grow the cells to the correct density necessary for competency. The competent cells were stored at -80°C.

May 9
We had a big group brainstorming session to identify goals and assays for the summer. The chassis came up with four goals: Transform and integrate our constructs into *Bacillus*; find the optimal media for cell growth in a patch; have sustainable expression of BBI; have BBI secreted by *Bacillus*. In general, the main experiments that we wanted to focus on are growth curves and cloning.
We used the competency test kit from igem to see how competent our cells were. 100ul aliquots of TOP10 and C3037 were transformed with the pSB1C3+RFP at the following concentrations: 0.5pg/ul, 5pg/ul, 10pg/ul, 20pg/ul, 50pg/ul. Transformation occurred following the transformation protocol.

May 10
Of the 10 aliquots transformed with pSB1C3 containing RFP, there were three Chlor plates that contained growth. Two plates that grew had been transformed with 5 pg/ul and the third plate was a concentration of 0.5 pg/ul. All of the bacteria that were transformed were *E. coli* TOP10. There was a lawn of growth of clear and colourless bacteria on both the Chlor and Amp plates. The Amp plates were

for the negative control. The strange growths are a sign of contamination and that the concentration of Amp is too low.

A colony PCR was run to check that the plasmids we hopped to transform into *E. coli* were actually there. There was an issue with the strip tubes, apparently the lids had not been closed all the way. Resulting in evaporation of the liquid in all but 4 of the tubes. The last step was to confirm the PCR with a agarose gel. A 1% agarose gel was prepared according to the gel electrophoresis protocol. 2 lanes were run with 1KB plus DNA ladder from AMRESCO.

Two overnight cultures of 5 ml each were generated from each of the transformed plates. The tube were incubate at 37℃ with shaking at 200rpm.

New Amp plates were poured but with a greater concentration of Amp, 100 ug/mL.

May 11

cPCR was repeated but with colonies with from the two master plates made from the *E. coli* TOP10+RFP. Twelve samples total used.
The following conditions were used: 3 min @ 95℃; 30 sec @ 95℃; 30 sec @ 62℃; 30 sec @ 72℃ and repeat the last three steps 33 times; 7 minutes at 72℃. The cPCR was then confirmed with a gel. Unfortunately, there did not seem to be amplification of the RFP gene. There were no bands present on the gel.

In the meantime, glycerol stocks of the *E. coli* and the pSB1C3+RFP were made following the glycerol stock protocol. Stocks were stored at both -20℃ and -80℃.

May 12

We reacquainted ourselves with the procedure for restriction digests and ligations. We chose two registry parts from the kit plates to work with.

Part	Description	Kit	Plate	Well
BBa_J23100	Constitutive promoter	2013	5	18C
BBa_E0240	RBS+GFP	2014	2	24B

The DNA from the Registry kit plates were rehydrated with 10µL of ddH_2O and let to sit for ten minutes at room temperature. We could tell that the DNA was being mixed due to the red solution in the well. We followed the rehydration protocol.

The two different parts were digested separately following the protocol Dan gave us for restriction digests. The promoter was digested with PstI and SpeI; RBS+GFP was digested PstI and XbaI. These enzymes were chosen as to create a scar between the different constructs. The scar removes any restriction site, permanently binding the two constructs together. The reactions were incubated at 37℃ for an hour followed by twenty minutes at 82℃ and stored at -20℃

Outside of the wet lab, planning started on the genetic circuits. In the operon we wanted a strong promoter (pGrac), rbs, and a double terminator (BBa_0010).

May 14

The restriction digest products were ligated together following the ligation protocol from Dan. 7µL of vector and 5µL of inset were used with the appropriate volume of buffer, water, and ligase. The reaction was incubated at room temperature for one hour.

Following the ligation, the products were used to transform *E. coli* TOP10 following the transformation protocol. The *E. coli* was plated on both Chlor and Amp plates. Amp was used as the negative control and to test the viability of the Amp plates. The *E. coli* were incubated overnight at 37°C.

Overnight cultures of the *E. coli* TOP10 transformed with the pSB1C3+RFP from May 9th were made in LB media. The cultures were grown overnight at 37°C with shaking at 200rpm.

May 15
The transformation results were observed. There were no colonies on the Amp plates, a good sign that the new concentration of Amp was sufficient. There were colonies on the Chlor plates but there was no visible GFP in natural or U.V light.

Using the overnight cultures from yesterday, we familiarized ourselves with the plasmid extraction (miniprep) protocol. The plasmids were extracted using the E.Z.N.A Plasmid Mini Kit (Omega Bio-Tek) and following its protocol. Three sample of the pSB1C3 were minipreped. Plasmid yield was checked using nanodrop spectrophotometer at an absorbance of 260nm. The caveat with this method is that the genomic and plasmid DNA cannot be differentiated based of A_{260}. The yields were quite low: 1.7, 3.1, and 62.7ng/ul. The purity of the plasmids was also quite bad, according to the $A_{260/280}$ value.

We rechecked our Chlor plates to see if the concentration of Chlor was sufficient. 50ul of untransformed *E. coli* was plated onto a Chlor plate and incubated at 37°C overnight.

May 16
Today we took care of minor housekeeping issues, 500mL of LB broth and 1000mL of LB agar. These were made following the recipes for broth and agar. 0.45mL of 50mg/uL chloramphenicol was added 750mL of agar while 250mL of agar was utilized for plain plates.

cPCR practice continued with *E. coli* TOP10 transformed with pSB1C3+RFP. A 4.5 times master mix was made for the four colonies selected from the plates. Volume of master mix used was 17uL with a total reaction volume of 25uL. Prefix and suffix primers were used. The same PCR conditions were used from May 11th.

May 17
Repeated yesterday's cPCR but with four different colonies. Unfortunately, there were no bands on the gel that we ran to confirm the pcr. Why tried to troubleshoot our protocol and the best explanation was that the annealing temperature was too high, preventing the primers from binding.

We got new *E. coli* TOP10 cells from Deirdre and streaked the cells onto plain agar plates and incubated them at 37°C overnight.
May 18
There was a lawn of growth of was observed on the plate that was spread with the *E. coli* TOP10.

May 19
We found *B. subtilis* spores from the 2014 iGEM team. We tried to germinate the spores by rehydrating the spores in 200ul ddH_2O. 50 ul of the resuspended spores were plated on plain lb plates while another 50ul was added to two overnight cultures. Both the plates and culture tubes were left to incubate at 37℃.

New glycerol stocks of *E. coli* TOP10 were made from the single colonies that were isolated from the cells that Deirdre gave us. The stocks were made following the glycerol stock protocol and stored at -20 and -80℃.

Since our gel worked yesterday, we decided to gel extract the band. The band we extracted was found at 1200 base pairs. The band was extracted using the Omega-Biotek E.Z.N.A gel extraction kit and following its protocol. Since we wanted to send the extracted DNA down to get sequenced, we discovered that we could not elute in elution buffer. The sequencing people demand that the DNA is only in ddH_2O. When we did elute the DNA from the column the yields were quite low, 3-5ng/ul, according to the nanodrop. Following Rai's advice, we added either elution buffer or water to the column, 30ul, and heated the column at 70℃ for 15 minutes. We then respun the columns. The yield improved dramatically, 22-43.2ng/ul.
The extracted plasmids were then prepared for sequencing following the sequencing lab's instructions. In a 0.2ml tube the following were mixed:

- 50-100ng of DNA for every 1000bp
- 3.2pmol of primer
- ddH_2O to 12ul total volume.

It is important to include the total plasmid length when determining how much template is needed.

May 20
After about a 18 hours of incubation, there was no growth or germination from the spores. This really wasn't that surprising since we don't know what has been done to these spores over two years ago. We left them t incubate over the weekend just encase.

There was no growth on the Chlor plate that was streaked with the new *E. coli* cells that we got from Deirdre!

Apparently more testing of e. Coli and Chlor plates

May 24
The spores did not germinate over the weekend after being incubated at 37℃ and grown on LB media. The untransformed *E. coli* did not grow on the Chlor plates, which is what we wanted. This means that there is no contamination of either the plates or the cells.
We took care of minor housekeeping items such as making more TAE buffer. We also started to look around the lab for any reagents that we might have to make various medias in the future.

June 1
It's June!
We found more spores from the 2014 team in the fridge and in what we suspect is water. We plated 50ul of the culture onto plain agar plates and incubated them overnight at 37℃.

June 6
A gram stain was done to see what state the *B. subtilis* spores were in. A gram stain was used as the spores would not stain and appear colourless under the microscope, while the vegetative cells stain purple (gram positive). Looking at the results of the stain under the microscope, there were primarily spores that had not been stained. There were some purple, rod shaped cells but they weren't very clear. The magnification used was 40x. Similar results were seen when cultures were taken from the culture tubes rather than the plates.

June 7

One liter of LB agar and 500mL of LB broth were made following the recipe for LB media. The broth is for growth curves that will start tomorrow. We ended up having to redo the LB plates as we think someone washed the bottle containing the media with soap :(. To prep for the growth curves 4 tubes of 1 mL LB-Chlor with a loop of *E. coli* TOP10 and incubated at 37.5℃ overnight.

June 8
We started the first growth curve. We are working with *E. coli* to practice and get a sense of how bacteria grows. One milliliter of cultured bacteria from the overnights was added to two flasks of 30mL of LB broth with chloramphenicol. One flask was kept at 4℃ as the negative control while the other was grown at 37.5℃. Growth was quantified by optical density, measured at 600 nanometers. Measurements were taken every half an hour. After each hour, 250 uL of each culture was plated onto Chlor plates with dilutions of 1:1000, 1:10000, and 1:100000.

June 9
We counted the cells that grew on our plates. We had so much counting that we enlisted everyone in lab! The 1:1000 dilutions contained too many cells to while the higher dilutions were reasonable. Based on the cell counts we realised that we needed to refine our approach to be sure we replicated the conditions in the patch as faithfully as possible.

More brainstorming was done to nail down our plan for growth curves. The biggest issue was that the OD values were above 0.4 for most of the experiment. Values over 0.4 are generally not considered accurate (for reasons). The next issue was how much to dilute the cultures when plating. To determine the best dilution, we made spot plates of the *E. coli* at dilutions from 1:10-1:1000000000.

Lastly we made LB plates doped with hygromycin to prepare for picking up *Bacillus subtilis* from Dr. Wong tomorrow.

June 10
Because our plates were not super dry, we had to leave the spot plates out overnight so that they could dry sufficiently before being placed in the incubator this morning.

To get a better idea of what temperature or patch will be at, we measured the different skin temperatures of everyone in the lab. Dr. Mayi was able to acquire a special thermometer from the U of C Medical Clinic. We found that the average skin temperature was around 35°C, which by a nice coincidence is the optimum temperature for *B. subtilis*. From this data, we decide to test growth at three temperatures: 4, 22, and 35°C. 22°C was selected as it is the average temperature of the International Space Station. This would be to test what would happen in if the spores germinated prematurely when in space.

We improved our plan for growth curves. We decided on four temperatures:

- 4°C negative control
- 22°C room temperature of ISS
- 35°C skin temperature
- 37°C positive control

We plan to inoculate 100mL of LB media with *E. coli* and every hour measure and spot growth and colonies respectively. With growth, we would be diluting the culture when the OD values climb above 0.4. Each run would take two days to do. The night before measurements start, media will be inoculated at 5:40pm and 12:20am. This is done in order to cover a 24 (48) hour time period within an 8 hour period.

Lastly, we thought of different ways we could test the viability of spores, the list was very extensive but it is a fairly low priority.

June 14
The *B. subtilis* spores germinated on the LB-Hygro plates! There was a band of growth around the filter paper. We then made an overnight culture of the freshly grown *B. subtilis* we incubated the *B. subtilis* at 30℃ instead of 37℃ as per Dr. Wong's instructions. An overnight culture of TOP10 was set up in chloramphenicol media and incubated at 37℃.

June 15
The streaked LB plate from yesterday was taken out of the incubator at precisely 9:30 am so that glycerol stocks of the *B. subtilis* could be made with the cells in log phase rather than stationary. Dr. Wong assured us that this would produce glycerol stocks with higher cell concentrations than our usual method. The glycerol solution was 250ul of plain LB with 250ul of 100% glycerol leaving us with 50% glycerol (v/v). Colonies were lifted from the plate using the streak loop. The new stocks were stored at -80℃. A new *B. sub* streak plate was made on hygromycin agar as the new master plate.

The TOP10 overnight culture was minipreped using the E.N.Z.A Plasmid mini kit from Omega. We followed the spin protocol with two modifications (1) columns were washed with 300uL of DNA wash in order to prep the columns and (2) prior to the elution step, the columns were heated at 70℃ for 15 minutes. This was done in the hope that it would boost the low yields we had been seeing. When the DNA was eluted, the yields were quite low, 4.4 and 5.2ng/uL respectively. Purity values were also poor: 260/280= 1.71 and 1.35; 260/230= 0.35 and 0.30.

We asked Deirdre what could be done to improve our persistently low yields. She told us two things: first was that we should have a longer lysis stage with more continuous inversion; second was to elute twice with 30uL of elution buffer after letting the buffer sit in the column.

New overnights of TOP10 started for more minipreps tomorrow.

June 16
The minipreps were done using the new Omega-Biotek plasmid mini kit that we ordered as well as the Sigma Aldrich Genelute that Deirdre lent to us. Protocols from each kit were followed.
The results are the following:

Sample	Concentration	A260/280
EZNA Omega Bio-Tek	27.5 ng/uL (in 100 uL)	1.78
Sigma Aldrich	37.2 ng/uL (in 50 uL)	1.84

DNA from IDT (*E. coli* BBI Construct) was rehydrated using the protocol found on their website:

1. Spin down for 3-5 seconds
2. 20ul ddH2o added to get a concentration of 50ng/ul (5x stock). The official protocol is to add enough water to reach a concentration of 10ng/ul. TE buffer was the recommended for rehydration as it is better for long term storage which is not a concern for us.
3. Tube was vortexed
4. Incubated for 20 minutes at 50℃
5. Store at -20℃

Both the extracted plasmid and rehydrated linear DNA were digested using EcoRI and PstI using the protocol from the 2014 Ucalgary iGEM team. The digests were incubated for 1 hour at 37℃ and then 20

minutes at 80℃. Following the digest, the products were ligated together using the T4 ligase from NEB, following the protocol again from 2014 Ucalgary iGEM team.

June 17

More DNA from IDT was rehydrated (BBI 5-GFP for *E. coli*) following the protocol used the previous day. 1.65ul of 50ng/uL of BBI-GFP and 1ul of 27.5ng/uL of pSB1C3+RFP were digested with EcoRI and PstI following the restriction digest protocol. Products of the digestion were ligated again following the ligation protocol.

The solutions for our new homemade miniprep kit were made:

- P1: 50mM Tris-HCl pH 8, 10mM EDTA, 100ug/mL RNase A
- P2: 200 mM NaOH, 1% SDS (V/V)
- P3: 3.0M potassium acetate pH 5.5

N.B: The EDTA will not dissolve in water until it is in a basic solution. It will then need to be pH corrected. The potassium acetate will also need to pH corrected as well.

June 20

Mostly Housekeeping things were done. New master plate of TOP10 was made on a plain agar plate and incubated at 37℃ overnight. We also tested glycerol stocks that we found were stored in -20℃ rather than -80℃. A loop of TOP10 stock was plated on plain lb and incubated overnight at 37℃. And to check whether the competent cells were still competent, they were transformed with BBI-GFP as a test. The transformed cells were plated on Chlor plates.

June 21

Not much happened today, more competent cells and glycerol stocks were made. The an aliquot of competent cells were used as a test batch. The aliquot was transformed with BBI-GFP in the pSB1C3 vector.

June 22

Start of the improved growth curve experiments. Three different 50mL falcon tubes with 9mL of hygromycin media were inoculated with 1mL of overnight *B. subtilis* culture at three different time points: 4:40pm, 12:20am and 8:00am. This was to cover 24 hours of growth in one work day. Growth was measured by a spectrophotometer at an optical density of 600nm. Each sample was measured every hour for eight hours. 100uL of culture was removed for measurement and diluted 1:10 in fresh media. This was to prevent the OD from climbing above 0.4. The results are as follows:

Time (h)	T=4C	T=22C	T=35C
0	0.029	0.01	0.011
1	0.034	0.036	0.028
2	0.006	0.029	0.043
3	0.016	0.008	0.059
4	0.009	0.01	0.12
5	0.005	0.014	0.171

6	0.002	0.02	0.196
7	0.014	0.036	0.217
8	0.04	0.047	0.308
9	0.031	0.089	0.293
10	0.009	0.084	0.292
11	0.017	0.084	0.374
12	0.005	0.1	0.392
13	0.001	0.093	0.395
14	0	0.108	0.427
15	0.009	0.12	0.394
16	0.029	0.148	0.384
17	0.029	0.184	0.384
18	0.006	0.16	0.39

Time (h)	T=4C	T=22C	T=35C
19	0.006	0.167	0.376
20	0.001	0.176	0.368
21	0	0.182	0.379
22	0	0.201	0.344
23	0.006	0.171	0.335
24	0.003	0.205	0.303

At times 0 and 24, dilutions of 1:1000, 10000, 100000, and 1000000 were made to determine the initial and final concentration of colony forming units. The dilutions were plated on agar plates and incubated overnight at 37°C. The resulting colonies were counted.

Temperature and Time	1:1,000 dilution	1:10,000 dilution	1:100,000 dilution	1:1,000,000 dilution
T=4C, T0	560	51	9	3
T=4C, T24	688	111	7	3
T=22C, T0	504	113	18	5

T=22C, T24	TNTC	2264	827	136
T=35C, T0	249	17	2	2
T=35C, T24	TNTC	TNTC	783	103

June 23

To try and boost the amount of linear DNA we had to work with, pcr of the rehydrated IDT BBI-GFP construct was carried out. This was done following IDT's protocol for PCR. Unfortunately, there were bands on the subsequent 1% agarose confirmation gel. We think the issue was that the annealing temperature was too high.

The pSB1C3-RFP plasmid that was transformed into TOP10 was minipred using the homebrew miniprep kit for the first time! And it worked! The yield was 36.6ng/uL with an A260/280 value of 1.90. The values were obtained from the nanodrop.

June 24

TOP that was transformed with BBI-GFP (June 21) was streaked onto Chlor plates and inoculated in Chlor broth. Both were left to grow overnight at 37°C. Another round of minipreps were done with the homebrew kit to extract pSB1C3-BBI #5-GFP. The end concentration was 63.6ng/uL with a 260/280 value of 1.93.

The PCR amplification of RFP was attempted but using the KAPA HiFi protocol. This makes more sense given that our reagents were from kapa and the protocol would be optimized for the reagents. The RFP was used as a control to make sure that we had the right annealing temperature. The PCR was run using the following set up:

PCR steps:

2 min at 95C		1 cycle
30 s at 98 C	}	
30 s at 52C	}	30 cycles
1 min at 72C	}	
5 min at 72C		1 cycle

On a 1% agarose gel that was run to confirm the products, a band was seen at ~1600bp relative to the 1 kb plus ladder.

June 27

Using the transformed TOP10 we tried to confirm that BBI-GFP had been inserted into the bacteria, accomplished by colony PCR. Also amplified was the BBI-GFP now that the correct temperatures had been found. The same run settings were used as the previous day. There were eight samples amplified and then run on a 1% agarose gel for confirmation.

Lane 1	ladder
Lane 2	colony 1
Lane 3	colony 2
Lane 4	colony 3
Lane 5	colony 4
Lane 6	linear BBI #5-GFP
Lane 7	linear BBI #5-GFP
Lane 8	plasmidal BBI #5-GFP
Lane 9	plasmidal BBI #5-GFP

The strongest band observed was at 2100bp which was the size of our plasmid. The lanes with linear DNA only resulted in smears, which suggests either contamination or off target binding.

June 28

BBI #5-GFP in the pSB1C3 vector was sent down to the sequencing lab to be sequenced. Samples had to be prepared to the exacting standards of the sequencing lab. For each sample 3uL of template at 63.1ng/ul was used along with 1ul of Prefix-F or Suffix-R. The tubes were filled up to 12uL volume with ddH_2O.

More glycerol stocks of BBI #5-GFP were made.

June 28-July 1

Another round of growth curves was done over the course of four days. Bacteria was grown again at four different temperatures with growth being measured at hour time points.

Time (h)	T=4C	T=4C	T=22C	T=22C	T=35	T=35
0	0.003	0.005	0.004	0.003	0.01	0.024
1	0.002	0.003	0.008	0.008	0.014	0.029
2	0	0	0.009	0.005	0.034	0.069
3	0	0	0.011	0.006	0.06	0.136
4	0.0063	0.006	0.013	0.01	0.137	0.152
5	0.0055	0.0119	0.019	0.018	0.182	0.182
6	0.0059	0.0071	0.03	0.025	0.152	0.199
7	0.0064	0.0064	0.05	0.037	0.218	0.228
8	0.0058	0.007	0.0595	0.0575	0.2005	0.2655
9	0	0	0.076	0.098	0.222	0.246
10	0	0	0.103	0.113	0.264	0.271
11	0	0.0045	0.107	0.138	0.293	0.275
12	0.0039	0.0042	0.146	0.154	0.316	0.334
13	0.0038	0.0038	0.174	0.192	0.346	0.351
14	0.0043	0.004	0.208	0.206	0.367	0.368
15	0.0042	0.0046	0.193	0.227	0.397	0.56
16	0.0037	0.0041	0.2545	0.248	0.395	0.383
17	0	0	0.276	0.296	0.347	0.396

18	0	0	0.295	0.278	0.371	0.45

Time (h)	T=4C	T=4C	T=22C	T=22C	T=35C	T=35C
19	0.0049	0.0039	0.325	0.295	0.364	0..38
20	0.0032	0.0032	0.333	0.311	0.355	0.74
21	0.0033	0.0033	0.326	0.317	0.325	0.349
22	0.0041	0.0037	0.355	0.324	0.37	0.345
23	0.0038	0.0039	0.363	0.332	0.346	0.315
24	0.0034	0.0043	0.359	0.340	0.335	0.35

As per the first growth curve dilutions were made to determine the initial and final CFU concentration.

Temperature and Time	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
T=4C, T0	N/A	1180	177	7	1
T=4C, T0	N/A	1179	191	9	4
T=4C, T24	TNTC	711	63	N/A	N/A
T=4C, T24	TNTC	751	52	N/A	N/A
T=22C, T0	N/A	N/A	26	6	N/A
T=22C, T0	N/A	N/A	53	9	N/A
T=22C, T24	N/A	N/A	TNTC	267	52
T=22C, T24	N/A	N/A	TNTC	342	135
T=35C, T0	TNTC	904	75	N/A	N/A
T=35C, T0	TNTC	1960	172	N/A	N/A
T=35C, T24	N/A	N/A	1460	299	12
T=35C, T24	N/A	N/A	752	199	31

We tried to amplify BBI #5-GFP for the third time using the KAPA HiFi protocol. However VF2 and VR primers were used instead of the standard biobrick primers to see if the construct would be amplified from the pSB1C3.
The products were run on a 1% agarose gel for 45 minutes.

June 29

Stocks of ECE 103 and 153 arrived from Bacillus Genetic Stock Centre and overnight cultures of both were prepared in LB-Amp broth. Lots of media was made to account for the Amp and Kan markers used on these plasmids.

June 30
Glycerol stocks of the ECE 103 and 153 were made following standard protocol.
The integration vectors were mini preped from the two different *E. coli* strains.

E. coli strain	integration vector	concentration (ng/uL)	A260/280
ECE 103 (sample 1)	pMLK83	111.3	1.92
ECE 103 (sample 2)	pMLK83	146	1.84
ECE 153 (sample 1)	pSG1154	78.5	1.84
ECE 153 (sample 2)	pSG1154	188.4	1.89

Rai came by to help us amplify our constructs to try and fix what was going wrong. Both the RFP and BBI #5-GFP were amplified with a variety of different primers: BBK primers, biobrick standard, and verification primers. Following the amplification, the products were run on a 1% agarose gel. Also included was a confirmation digest of the pSB1C3 plasmid with RFP and BBI #5-GFP to ensure that it hadn't degraded. We followed Rai's procedure for that.

4 uL template
1 uL BSA
1 uL EcoRI
1 uL PstI
2 uL buffer (X10)
17 uL ddH2O
These samples were left to incubate at 37℃ for 1 hour before heat shocking at 80℃ for 20 min

Lane assignments for PCR/digestion products

Lane	GFP Gel	RFP Gel
1	BBI #5GFP using BBK primers	RFP using BBK primers
2	BBI #5GFP using BBK primers	RFP using BBK primers
3	BBI #5GFP using BBK primers	RFP using BBK primers
4	BBI #5GFP using BBK primers	RFP using BBK primers
5	Ladder (GeneRule 1Kb Plus)	Ladder (GeneRule 1Kb Plus)
6	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers

7	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers
8	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers
9	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers
10	Ladder (GeneRule 1Kb Plus)	Ladder (GeneRule 1Kb Plus)
11	BBI #5GFP using Verification primers	RFP using Verification primers
12	BBI #5GFP using Verification primers	RFP using Verification primers
13	BBI #5GFP using Verification primers	RFP using Verification primers
14	BBI #5GFP using Verification primers	RFP using Verification primers
15	pSB1c3-BBI #5GFP restriction digest	pSB1c3-RFP restriction digest

GFP Gel the results are as follows:

- BBK Primers: bands were seen at 1300 bp (expected = 1250 bp) and 300 bp
- Prefix and Suffix Primers: bands were seen at 1100 bp (expected = 970 bp)
- VerificatioPrimers: faint bands were seen at 1300 bp (expected = 1221 bp) and 400 bp
- Restriction digest: one faint band was seen at 2100bp (not enough DNA was present)

RFP results

- There were no bands

We got a new strain of *E. coli* from Deirdre: DB3.1. We wanted to use this strain as it is resistant to the *ccdB* gene which is a component of the new pSB3C5 (low copy number) plasmid we want to use. Low copy number hopefully will mimic having the BBI integrated into the bacillus genome at several different positions. We don't have the time or the expertise to integrate multiple different constructs into the *Bacillus* genome. The bacteria were streaked onto an LB plate and incubated overnight at 37°C.

July 4

Overnight cultures of *E. coli* DB3.1, ECE103. ECE153, RFP and BBI #5GFP were prepared in 7mL of LB, LB-Amp, -Chlor respectively. The overnights were incubated at 37°C with shaking at 200rpm.

Master plates of *E. coli* DB3.1, ECE103 and ECE153 were plated stored at 4°C.

Dilutions of 1:1, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000 of frozen *B. subtilis* samples s3 taken at time T8 at temperatures of 4°C, 22°C and 35°C were spot-plated (20uL) on LB plates and left to incubate O/N at 37°C to determine the presence of spores in each sample.

We digested pSB1c3-RFP, pSB1c3-BBI #5GFP, pMLK83 and pSG1154 using Rai's protocol.

	BBI #5GFP	RFP	pMLK83	pSG1154
single digest	PstI	PstI	HinDIII	HinDIII
"double" digest	PstI + EcoRI	PstI + EcoRI	EcoRI	XbaI

The digested products were run on a 1% agarose gel and run at 120 V for 45 min:

Lane	Contents	Expected bands (Kb)	Observed bands
1	1 Kb Plus GeneRuler ladder		none
2	pSB1c3-BBI #5GFP/PstI	3.1	none
3	pSB1c3-RFP/PstI	3.2	none
4	pMLK83/HinDIII	9.9	none
5	pSG1154/HinDIII	7.6	none
6	pSB1c3-BBI #5GFP/PstI+EcoRI	1, 2.1	none
7	pSB1c3-RFP/PstI+EcoRI	1.1, 2.1	none
8	pMLK83/EcoRI	2, 7.9	none
9	pSG1154/XbaI	1, 1.6, 5	none

July 5
The frozen spores from yesterday grew and 1:10000 dilution is the most appropriate for spot plating.

The DB3.1 cells that we obtained from Deirdre were made competent following the chemical competency protocol for *E. coli*.

Using the overnight cultures: pSB1c3-BBI #5GFP, pSB1c3-RFP, pMLK83 and pSG1154 were double digested with EcoRI and XbaI while pSB1C3 with RFP or BBI #5-GFP were singly digested with EcoRI as well.

July 6
pSB1c3-BBI #5GFP, pSB1c3-RFP, pMLK83 and pSG1154 were prepped from *E. coli* using the Omega and homebrew kits (protocol on page 3 of Rachelle's lab book) for comparison. The plasmid yield was determined with the nanodrop spectrophotometer.

Plasmid	Omega concentration (ng/uL)	Omega A260/280	Homebrew concentration (ng/uL)	Homebrew A260/280
pSB1c3-BBI #5GFP	45.7	1.89	171.1	1.86
pSB1c3-RFP	94.6	1.86	120.4	1.85

pMLK83	38.9	1.86	99	1.85
pSG1154	20.9	2.16	137.1	1.87

The plasmids were the sent for sequencing. We sent down 6 samples of pSB1c3-BBI #5GFP; 3 samples contained the forward Prefix-F primer and 3 samples contained the reverse Suffix-R primer. However, we hit a snag :(We need to reapply for our sequencing authorization, so we're waiting for Dr. Mayi to come in from her vacation to sign forms.

The restriction digests from yesterday (July 5) and the minipreps from today were run on a 1% agarose gel at 120 V for 45 min:

Lane	Contents of well	Expected band size (kb)	Observed band size (kb)
1	GeneRuler 1KB Plus ladder		
2	pMLK83 from Omega kit digest: EcoRI+XbaI	2, 7.9	2, 7.5
3	pSG1154 from Omega kit digest: EcoRI+XbaI	1, 1.6, 5	1.5, 5
4	pSB1c3-RFP from Omega kit digest: EcoRI + XbaI	3.1	3.1
5	pSB1c3-BBI #5GFP from Omega kit digest: EcoRI + XbaI	3.1	3.1
6	pSB1c3-BBI #5GFP from homebrew digest: EcoRI	3.1	3.1
7	pSB1c3-BBI #5GFP digest: EcoRI	3.1	3.1
8	pSB1c3-RFP from homebrew digest: EcoRI	3.1	>20
9	pSB1c3-RFP from homebrew digest: EcoRI	3.1	>20
10	pSB1c3-BBI #5GFP undigested from homebrew	?	2.5
11	pSB1c3-RFP undigested from homebrew	?	6
12	pMLK83 undigested from homebrew	?	10
13	pSG1154 undigested from homebrew	?	7
14	GeneRuler 1KB Plus ladder		

Almost everything worked! We reprepped the RFP plasmid...

We transformed the DB3.1 cells with the pSB3C5 plasmid that was in well 4D of kit plate four in the 2014 distribution kit.

Two different types of media were made in preparation for the next set of growth curves

2X LB Media - in 1 L dH2O

2% tryptone (20 g)
1% yeast extract (10 g)
2% NaCl (20 g)

Super-Rich Media - in 1 L dH2O
2.5% tryptone (25 g)
2% yeast extract (20 g)
0.3% K2HPO4 (3 g)
3% lucose (150 mL of 20%)
Mix all components except glucose and adjust pH to 7.5
Autoclave solution except glucose
Filter sterilize glucose and add to cooled solution
If pH<7, add 1.5 mL of 6M NH4OH per litre to raise pH

July 7

A restriction digest of pMLK83 and pSG1154 was performed in separate reaction tubes. pMLK83 was digested using EcoRI and XbaI and pSG1154 was digested using EcoRI and SpeI following Rai's protocol (on page 31 of Rachelle's lab book); **we realized too late that the digest of pMLK83 wouldn't allow for our insert to be ligated because both EcoRI and XbaI are in the prefix**...

Another confirmation digest was run for the four plasmids we were working with: -pMLK83, pSG1154, pSB1c3-RFP and pSB1c3-BBI #5GFP. The products were then run on a 1% gel

Lane	Contents of the well	Expected band size (kb)	Observed band size (kb)	Explanation
1	1KB Plus GeneRuler ladder			
2	pMLK83 digested with PstI	4.4, 5.5	4.4, 5.5	
3	pMLK83 digested with PstI	4.4, 5.5	4.4, 5.5	
4	pSG1154 digested with PstI	3.4, 4.2	4.2, 7.6	nicked plasmid => 7.6 kb, 3.4 was too faint to visualize
5	pSG1154 digested with PstI	3.4, 4.2	4.2, 7.6	nicked plasmid => 7.6 kb, 3.4 was too faint to visualize
6	pSB1c3-RFP digested with PstI+EcoRI	1.1, 2.1	3.1	no EcoRI was added

7	pSB1c3-RFP digested with PstI+EcoRI	1.1, 2.1	1.1, 2.1	
8	pSB1c3-BBI #5GFP digested with PstI+EcoRI	1, 2.1	3.1	no EcoRI was added
9	pSB1c3-BBI #5GFP digested with PstI+EcoRI	1, 2.1	1, 2.1	
10	1KB Plus GeneRuler ladder			

All 4 plasmids were confirmed! Yay!

July 8
We finally made transformation media. We first made stock solutions of everything and autoclaved them except for the glucose, which was filter sterilized. We followed the recipe provided to us by Dr. Wong.

We realized that digesting the *ComK* insert with EcoRI and XbaI wouldn't work as both of the restriction sites are in the prefix region. The digestion was redone, following Rai's protocol. The products were then ligated into the pMLK83 vector following the usual protocol.

July 9
The media test growth curves have started! A 7mL of LB-hygro was inoculated with *B. subtilis* at 4:45pm and incubated at 37°C and 200rpm overnight.

2 aliquots of TOP10 were transformed he newly-ligated pMLK83-*ComK* and pSG1154-*ComK* plasmids following the standard procedure. 10ul of each DNA sample was used and the transformed cells were plated on Amp plates. The bacteria were then grown up overnight at 37°C

July 11
We started measuring the growth of *B. subtilis* but every 12 hours we added a 1mL of media. The addition of media was to simulated popping one of the additional media packets in the patch. Each round of growth curves a different type of media was added. The experiments were set up the same as the previous growth curves. A 50mL falcon tube with 9mL of hygromycin media was incubated with 1 mL of culture and grown at 35°C and 200rpm. The four different treatments were: nothing added, plain LB, 2x concentration of LB, and super rich media. As before, at the start and end of the experiments, dilutions of the culture are made to determine initial and final CFU (colony forming units).

Adding super rich media every twelve hours:

Time (h)	0-8 h (1)	0-8 h (2)	8-16 h (1)	8-16 h (2)	16-24 h (1)	16-24 h (2)
T0	0.026	0.020	0.146	0.132	0.208	0.233
T1	0.031	0.028	0.170	0.161	0.244	0.313
T2	0.053	0.041	0.183	0.177	0.282	0.389
T3	0.099	0.098	0.288	0.215	0.263	0.312

T4	0.113	0.153	0.239	0.249	0.240	0.336
T5	0.142	0.126	0.255	0.245	0.324	0.342
T6	0.181	0.175	0.243	0.289	0.337	0.380
T7	0.192	0.187	0.293	0.297	0.338	0.339
T8	0.226	0.221	0.355	0.309	0.339	0.363

All OD measurments were made at 1:10 dilution unless otherwise stated.

July 12

A 1% agarose gel was run to confirm that the extracted plasmids were not degrading while in storage. The four plasmids digested were: pMLK83, pSG1154, pSB1C3+BBIGFP, and pSB1C3+RFP. All plasmids were singly and doubly digested. The ladder used was the GenRuler 1KB plus and the gel was run for 45 minutes at 120V. The gel ran quite weirdly, bands seemed to be off and the ladder looked like it had been degraded. The gel was re-done just in case.

We tried to PCR out Comk from pMLK83 and pSG1154 transformed into TOP10. A 25ul reaction protocol was used instead of 50ul. 8 colonies from each plate were selected for 16 total reactions and 17x master mix. The KAPA PCR protocol was used but adjusted to the smaller volume.On the confirmation gel, there were no bands for either of the pMLK83 or SG1154. The ladder used was the GenRuler 1kb plus which was very faint.

Growth curves with super rich media continues:

Time (h)	Tube 1 24-32 h (1)	Tube 2 24-32 h (2)	Tube 3 32-40 h (1)	Tube 4 32-40 h (2)	Tube 5 40-48 h (1)	Tube 6 40-48 h (2)
T9	0.071	0.056	0.063	0.060	0.058	0.054
T10	0.041	0.048	0.039	0.040	0.037	0.048
T11	0.045	0.050	0.047	0.066	0.044	0.048
T12	0.047	0.061	0.044	0.047	0.050	0.059
T13	0.086	0.065	0.050	0.049	0.049	0.058
T14	0.054	0.063	0.041	0.069	0.058	0.058
T15	0.059	0.69	0.057	0.047	0.041	0.055
T16	0.053	0.072	0.049	0.066	0.067	0.068
T17	0.055	0.058	0.045	0.050	0.052	0.050

*all measurements are made in a 1:100 dilution unless stated otherwise

Initial Cell Count - CFU per 100 uL

CFU (in 100 uL)	**1:1,000 dilution**	**1:10,000 dilution**

Tube 1, T0	TNTC	522
Tube 2, T0	TNTC	424

July 13

More colony PCR of the TOP10 transformed with pMLK83+*ComK* and pSG1154+*ComK*. Colonies 9-24 were selected on both plates for a total of 32 reactions and master mix of 33x. Same protocol as yesterday. The products were run on gel and the bright bands ⅔ of the way down the gel are approximately 700-800bp (above the empty space where the loading dye is). There is another series of bands at the 1000-1200 bp range. The second series of bands is the range of interest as *ComK* is ~1200 base pairs. The large amount of smaller bands is worrying as it indicates that the primers could be annealing to off-target sites. We aren't sure as the plasmid sequences are not available.

The pSB3C5, which contained BBa_I52001 (*Cccd*), was minipreped from DB3.1 following the homebrew protocol. The yield was 545.7ng/ul with a A260/280 of 1.82.

All the plasmids were digested again because we are paranoid that the plasmids might be being degraded by a nucleases in the water we were using for resuspending the pellet during minipreps. We followed Rai's protocol and ran the products on an 0.8% agarose gel at 100v for 45 minutes. All of our plasmids are now confirmed.

Lane	Well contents	Expected bands (kb)	Observed bands (kb)
1	1 Kb Plus GeneRuler DNA ladder		
2	pMLK83 digested with XbaI	9.9	9,9
3	pSG1154 digested with EcoRI	7.6	7.6
4	pSB1c3-RFP digested with EcoRI	3.1	3.1
5	pSB1c3-BBI #5GFP digested with EcoRI	3.1	3.1
6	pSB3c5-I52001 digested with EcoRI	3.8	3.8
7	pMLK83 digested with EcoRI + PstI	2, 3.5, 4.4	2, 3.5, 4.4
8	pSG1154 digested with EcoRI + PstI	3.4, 4.2	**2.5, 5**
9	pSB1c3-RFP digested with EcoRI + PstI	1.1, 2.1	1.1, 2.1
10	pSB1c3-BBI #5GFP digested with EcoRI + PstI	1, 2.1	1, 2.1
11	pSB3c5-I52001 digested with EcoRI + PstI	1.1, 2.7	1.1, 2.7
12	1 Kb Plus GeneRuler DNA ladder		

Continuation of *B. subtilis* growth curve with super rich media:

Time (h)	Tube 1 48-56 h (1)	Tube 2 48-56 h (2)	Tube 3 56-64 h (1)	Tube 4 56-64 h (2)	Tube 5 64-72 h (1)	Tube 6 64-72 h (2)

T18	0.051	0.074	0.046	0.051	0.060	0.043
T19	0.056	0.076	0.047	0.050	0.058	0.042
T20	0.056	0.077	0.052	0.062	0.057	0.050
T21	0.071	0.085	0.058	0.060	0.060	0.043
T22	0.064	0.072	0.048	0.069	0.051	0.042
T23	0.060	0.074	0.046	0.088	0.097	0.038
T24	0.116	0.135	0.096	0.107	0.107	0.088
T25	0.120	0.142	0.095	0.095	0.100	0.085
T26	0.134	0.143	0.096	0.104	0.106	0.095

*all measurements are made in a 1:100 dilution unless stated otherwise

July 14

Due to issues with the integration vectors, pMLK83 and pSG1154, we tried to to ligate the linear *ComK* into pSB1C3 as a backup plan. The *ComK* construct contains integration sites flanked by the prefix and suffix sequences allowing it to still be able to integrate into the *Bacillus* genome.

The digestions were done following Rai's protocol with only 1ul of *ComK* to save materials and 12ng/ul of pSB1C3. The insert and vector were ligated together using the usual protocol. Nick and Miriam did the digest and restriction so that they knew what to do when James and Rachelle are away.

The ligated products were subsequently transformed into TOP10 following the standard transformation procedure. However the one hour recovery period occurred at 35°C instead of 37°C as the shaker had not warmed up enough. The 50uL of the transformants were plated onto LB-Chlor plates.
The last day of the super rich growth curve! First day of using 2xLB!

Time	2X LB 0-8 h (1)	2XLB 0-8 h (2)	2XLB 0-8 h (3)	SR 0-8 h (3)	2XLB 8-16 h (1)	2XLB 8-16 h (2)	2XLB 8-16 h (3)	SR 8-16 h (3)	2XLB 16-24 h (1)	2XLB 16-24 h (2)	2XLB 16-24 h (3)	SR 16-24 h (3)
T0	0.006	0.005	0.007	0.005	0.129	0.066	0.134	0.126	0.219	0.185	0.233	0.157
T1	0.014	0.009	0.007	0.004	0.138	0.093	0.151	0.151	0.244	0.193	0.247	0.228
T2	0.042	0.021	0.023	0.020	0.156	0.109	0.156	0.161	0.241	0.234	0.275	0.254
T3	0.077	0.058	0.069	0.055	0.158	0.122	0.163	0.163	0.269	0.235	0.276	0.259
T4	0.119	0.101	0.089	0.095	0.181	0.140	0.186	0.194	0.287	0.244	0.299	0.275
T5	0.117	0.11	0.11	0.1	0.17	0.15	0.17	0.19	0.287	0.240	0.291	0.294

		2	4	01	9	4	9	2				
T6	0.130	0.128	0.125	0.123	0.204	0.181	0.210	0.235	0.310	0.266	0.3915	0.317
T7	0.139	0.147	0.154	0.139	0.230	0.266	0.230	0.284	0.318	0.291	0.312	0.318
T8	0.161	0.167	0.167	0.234	0.245	0.228	0.247	0.343	0.347	0.304	0.367	0.330

*all measurements are made in a 1:10 dilution unless stated otherwise
Initial cell counts:

CFU (100 uL)	**1:1,000 dilution**	**1:10,000 dilution**
2XLB, (1), T0	TNTC	79
2XLB (2), T0	contaminated	43
2XLB (3), T0	397	50
SR (3), T0	TNTC	39

July 15
The transformations from yesterday didn't work, there was no growth on any of the plates. We replated the transformants but used 100uL instead of 50uL. The cells were incubated at 37℃ overnight.

The last 11 colonies from the both the transformed MLK83 and SG1154 plates were PCRed to see if *ComK* was inserted into either of the integration vectors. The same procedure from July 12 was followed except that a 24x master mix was used instead of the 33x. The products were run on a 1% agarose gel for 25 minutes at 120v to confirm the PCR.

According to the bands on the gel, the primers amplified a small segment of dna less than 500bp in size. The second most prominent band is again around the 700 bp marker of the ladder. Where we expect to see *Comk*, 1200bp, is a very weak band. Again there appear to be primer issues lots of small products were generated including in the water control which should not have any DNA. The PCR products were run a second time on different gels to see if running a gel with only one well set would change the results. It didn't.

Continuation of Super Rich and 2xLB

Time	2xLB 1	2xLB 2	2xLB 3	SR 4	2xLB 5	2xLB 6	2xLB 7	SR 8	2xLB 9	2xLB 10	2xLB 11	SR 12
T9	0.262	0.316	0.295	0.300	0.307	0.282	0.303	0.073*	0.339	0.321	0.332	0.389
T10	0.234	0.271	0.251	0.280	0.326	0.273	0.302	0.0495*	0.307	0.320	0.31	0.365
T11	0.23	0.27	0.24	0.29	0.31	0.27	0.29	0.05	0.32	0.34	0.32	0.37

	9	5	5	3	4	6	7	3*	5	0	9	6
T12	0.275	0.286	0.243	0.340	0.331	0.288	0.308	0.070*	0.350	0.342	0.324	0.041*
T13	0.276	0.323	0.302	0.295	0.339	0.281	0.306	0.086*	0.052*	0.341	0.333	0.027*
T14	0.262	0.352	0.304	0.377	0.330	0.276	0.282	0.063*	0.053*	0.333	0.302	0.040*
T15	0.302	0.342	0.328	0.371	0.295	0.255	0.274	0.070*	0.029*	0.303	0.315	0.039*
T16	0.319	0.386	0.387	0.045*	0.307	0.304	0.323	0.055*	0.037*	0.331	0.339	0.052*
T17	0.323	0.354	0.325	0.049*	0.314	0.294	0.245	0.086*	0.035*	0.039*	0.289	0.066*

All measurements were at 1:10 dilutions apart from the *measurements which were at 1:100.

The DNA sequences for the indicator systems were rehydrated from a number of the kit plates. The parts rehydrated were BBa_K091110 and BBa_K909006 from the 2012 and 2016 kit plates respectively. The two parts were transformed into TOP10 with two aliquots each.

July 16

Time	2xLB 1	2xLB 2	2xLB 3	SR 4	2xLB 5	2xLB 6	2xLB 7	SR 8	2xLB 9	2xLB 10	2xLB 11	SR 12
T18	0.292	0.303	0.284	0.039*	0.261	0.356	0.300	0.074*	0.227	0.036*	0.189	0.031*
T19	0.268	0.301	0.283	0.053*	0.265	0.352	0.283	0.067*	0.225	0.056*	0.192	0.049*
T20	0.274	0.296	0.312	0.051*	0.266	0.360	0.300	0.066*	0.222	0.098*	0.201	0.029*
T21	0.268	0.291	0.279	0.057*	0.263	0.363	0.288	0.067*	0.209	0.075*	0.183	0.036*
T22	0.257	0.287	0.268	0.078*	0.266	0.351	0.300	0.072*	0.213	0.067*	0.216	0.008*
T23	0.258	0.283	0.289	0.083*	0.311	0.372	0.304	0.048*	0.276	0.135*	0.204	0.025*
T24	0.262	0.300	0.300	0.069*	0.284	0.375	0.314	0.066*	0.230	0.021*	0.208	0.019*
T25	0.24	0.28	0.26	0.05	0.25	0.36	0.30	0.06	0.22	0.07	0.18	0.05

	8	3	8	0*	4	3	2	6*	1	4*	9	9
T26	0.260	0.304	0.273	0.065*	0.256	0.333	0.263	0.100*	0.240	0.063*	0.226	0.045*

All measurements were at 1:10 dilutions apart from the *measurements which were at 1:100.

Bacillus subtilis was transformed with pSB1C3-RFP and pSB1C3-BBI #5GFP. The transformation protocol was provided by Dr. Wong. Between 120ng and 200ng of plasmid was used for the various transformations. 250uL of transformed cells were plated onto LB-Chlor plates and incubated overnight at 37°C.

July 18

No addition of media

Time	1	2	3	4	5	6	7	8	9
T0	0.002	0.002	0.004	0.139	0.142	0.153	0.226	0.232	0.245
T1	0.024	0.016	0.009	0.168	0.164	0.165	0.245	0.247	0.267
T2	0.015	0.002	0.001	0.166	0.184	0.192	0.278	0.285	0.302
T3	0.010	0.012	0.003	0.079	0.196	0.232	0.273	0.286	0.298
T4	0.012	0.013	0.001	0.187	0.221	0.241	0.308	0.323	0.326
T5	0.018	0.011	0.010	0.282	0.274	0.269	0.365	0.341	0.356
T6	0.011	0.005	0.011	0.244	0.309	0.313	0.383	0.373	0.374
T7	0.019	0.014	0.022	0.271	0.317	0.322	0.363	0.353	0.350
T8	0.013	0.013	0.041	0.241	0.328	0.354	0.362	0.364	0.383

All measurements were made at a 1:10 dilution

CFU (100ul)	1:1000	1:10 000
T0	70	41
T0	158	TNTC
T0	169	548

The *Bacillus* transformations were successful, there were lots of colonies growing on the LB-Chlor plates.

July 19

Time	1	2	3	4	5	6	7	8	9
T9	0.234	0.088	0.221	0.256	0.237	0.229	0.184	0.188	0.199

T10	0.199	0.180	0.255	0.245	0.238	0.224	0.202	0.200	0.195
T12	0.230	0.157	0.260	0.274	0.238	0.221	0.199	0.221	0.197
T13	0.265	0.186	0.283	0.223	0.211	0.196	0.199	0.224	0.179
T14	0.296	0.222	0.292	0.216	0.204	0.102	0.193	0.191	0.103
T15	0.313	0.226	0.289	0.218	0.215	0.189	0.169	0.190	0.170
T16	0.340	0.236	0.310	0.209	0.232	0.172	0.171	0.162	0.157
T17	0.353	0.256	0.203	0.171	0.179	0.170	0.156	0.151	0.157

All measurements were at a 1:10 dilution

pSB1C3-RFP and pSB1C3-BBIGFP were minipreped from the transformed *B. subtilis* and the pMLK83-*Comk* and pSG1154-*ComK* were minipreped from TOP10 using the homebrew miniprep kit.

Plasmid	Yield (ng/ul)	A260/280
pSB1C3-BBIGFP	50	1.50
pSB1C3-BBIGFP	96.1	1.56
pSB1C3-RFP	49.2	1.51
pSB1C3-RFP	40.7	1.57
pMLK83-*Comk*	59.9	1.74
pMLK83-*Comk*	240.2	1.90
pSG1154-*ComK*	334.2	1.89
pSG1154-*ComK*	373	1.84

The Plasmids were then digested to confirm their identity

Plasmid	Single digest	Double digest
pSB1C3-BBIGFP	EcoRi	EcoRi+PstI
pSB1C3-RFP	EcoRi	EcoRi+PstI
pMLK83-*ComK*	XbaI	EcoRi+PstI
pSG1154-*ComK*	EcoRi	EcoRi+PstI

Rai's protocol was used

July 20

Time	1	2	3	4	5	6	7	8	9

T15	0.278	0.294	0.247	0.168	0.160	0.158	0.131	0.131	0.159
T19	0.270	0.288	0.253	0.173	0.170	0.172	0.138	0.149	0.175
T20	0.250	0.270	0.244	0.145	0.153	0.132	0.135	0.146	0.152
T21	0.234	0.249	0.224	0.171	0.171	0.131	0.119	0.137	0.151
T22	0.249	0.260	0.234	0.165	0.152	0.152	0.121	0.120	0.148
T23	0.215	0.228	0.228	0.167	0.157	0.153	0.112	0.133	0.150
T24	0.220	0.228	0.221	0.164	0.154	0.148	0.132	0.126	0.159
T25	0.207	0.225	0.216	0.163	0.161	0.169	0.118	0.125	0.152
T26	0.206	0.218	0.210	0.160	0.151	0.144	0.116	0.120	0.147

All measurements were at a dilution of 1:10

The products of the restriction digests from yesterday were run on a 0.8% gel at 100v for 40 minutes. The vast majority of lanes had no bands and the lanes that did have bands were between 4 and greater than 10kb in size. Clearly these did not work.

July 21

Time	1	2	3	4	5	6	7	8	9
T0	0.004	0.001	0.002	0.003	0.003	0.089	0.191	0.131	0.169
T1	0.010	0.004	0.009	0.005	0.004	0.118	0.225	0.170	0.198
T2	0.011	0.003	0.004	0.001	0.001	0.135	0.236	0.182	0.212
T3	0.028	0.022	0.014	0.004	0.003	0.136	0.249	0.198	0.237
T4	0.061	0.046	0.045	0.001	0.010	0.165	0.263	0.208	0.236
T5	0.101	0.103	0.105	0.003	0.000	0.160	0.278	0.241	0.269
T6	0.133	0.117	0.117	0.006	0.003	0.189	0.292	0.212	0.265
T7	0.134	0.127	0.134	0.005	0.004	0.165	0.312	0.229	0.257
T8	0.170	0.156	0.155	0.011	0.004	0.182	0.295	0.242	0.262

All measurements were made at 1:10 dilution

July 22

Time	1	2	3	4	5	6	7	8	9
T9	0.171	0.149	0.153	0.162	0.119	0.243	0.203	0.202	0.199
T10	0.210	0.198	0.193	0.195	0.154	0.248	0.209	0.208	0.200

T11	0.234	0.209	0.211	0.228	0.194	0.258	0.199	0.204	0.188
T12	0.239	0.26	0.223	0.258	0.211	0.234	0.203	0.202	0.191
T13	0.300	0.246	0.245	0.278	0.59	0.251	0.191	0.186	0.175
T14	0.352	0.280	0.283	0.317	0.273	0.266	0.197	0.186	0.180
T15	0.332	0.264	0.278	0.291	0.223	0.209	0.162	0.161	0.158
T16	0.320	0.292	0.286	0.312	0.253	0.204	0.171	0.149	0.151
T17	0.311	0.272	0.271	0.313	0.276	0.186	0.157	0.150	0.144

July 23

Finished growth curves!

	1(0:00)	2 (:05)	3 (:10)	4 (:15)	5 (:20)	6 (:25)	7 (:30)	8 (:35)	9 (:40)
T0	0.236	0.170	0.146	0.191	0.201	0.084	0.084	0.089	0.077
T1	0.248	0.161	0.150	0.185	0.208	0.089	0.085	0.088	0.072
T2	0.241	0.157	0.137	0.150	0.209	0.093	0.089	0.088	0.076
T3	0.233	0.154	0.141	0.168	0.220	0.087	0.089	0.094	0.081
T4	0.222	0.151	0.136	0.172	0.244	0.088	0.093	0.091	0.085
T5	0.214	0.155	0.146	0.172	0.234	0.088	0.100	0.096	0.081
T6	0.215	0.152	0.143	0.162	0.201	0.077	0.086	0.088	0.073
T7	0.226	0.141	0.129	0.155	0.221	0.084	0.091	0.084	0.081
T8	0.206	0.137	0.123	0.158	0.201	0.077	0.099	0.091	0.076

July 25

Comk was digested and ligated into pSB1C3. The ligations were stored overnight at 4℃ in preparation for transformations the next day.

The BBI constructs for *Bacillus* were rehydrated following the IDT protocol. The final concentration was between 10-50uL. The constructs were then digested and ligated into pSB1C3 and then transformed into TOP10 which was then incubated overnight at 37℃.

July 26

The transformations with the *ComK* containing plasmids were inconclusive and we decided to redo everything the next day.

The BBI #1 with restriction enzyme sites (RE) and BBI #1 without RE transformations were successful, isolated purple colonies were seen for both. BBI #5 with RE and BBI #5 without RE transformations weren't successful, no colonies grew on the plates.

> Note: We revisited these results later in august and september and realised that these original transformations were actually not successful. The purple colour was due to RFP that was not cut out of the pSB1C3

Digestion, ligation, and transformation was repeated for the BBI #5 constructs. Lastly master plates for cPCR were made for both of the BBI #1 constructs.

July 27
Transformations of TOP10 with *ComK* inserted into the integration vectors was successful. The transformation with pSG1154+*ComK* may have not been successful given that there was only one colony on the plate. IT was discovered afterwards that pMLK83 and pSG1154 plasmids had Amp resistance and Chlor.

The *ComK* with *AmyE* integration sequences was rehydrated following the instructions from IDT. The rehydrated construct was then digested following Rai's protocol. 2-4uL of vector (pSG1154 and pMLK83 respectively) was used but only 2uL of insert was used to save on the limited amount of DNA we had to work with. Lastly, the construct in the TOP10 and plated on Amp. plates. A negative control of untransformed TOP10 was plated as well.

The BBI #5 transformations attempt #2 wasn't successful again. The plates were left in the incubator, in the hope that colonies would grow with more time. Colonies were observed after a while.

Finally, overnight cultures of BBI #1 with RE were made and incubated at 37℃.

July 28
We discovered that the concentration of Amp. was not strong enough as the negative control, untransformed TOP10 was able to grow after being plated yesterday. Thus, new Amp. plates were made with the new Amp. was delivered.

The overnight cultures from July 27 were minipreped using the homebrew kit. Five cultures were used:

Colony #	Plasmid	Yield(ng/ul)	A260/280
1	pSB1C3-BBI #1 RE	8.6	1.33
7	pSB1C3-BBI #1 RE	6.1	1.34
8	pSB1C3-BBI #1 RE	15.4	1.48
10	pSB1C3-BBI #1 RE	14.2	1.48
11	pSB1C3-BBI #1 RE	16.1	1.59

The other colonies (12-22) of the master plates for the TOP10 transformed with BBI #1 constructs were cPCRed and the products were run on the a 1% gel.
Master plates for the now grown BBI #5 constructs were made and incubated at 37℃ overnight.

July 29
Both of the BBI #5 constructs were cPCRed off the master plates from yesterday with the products run a gel for confirmation. Unfortunately there were no bands on the gel, maybe a problem with the PCR.

The same minipreps from yesterday were redone, again using the homebrew kit. Also included were two colonies from BBI #1 w/o RE

Colony #	Plasmid	Yield	A260/280
1	BBI 1 w/t RE	10.1	2.71
7	BBI 1 w/t RE	16.7	2.32
8	BBI 1 w/t RE	45.1	1.99
10	BBI 1 w/t RE	68.8	2.02
11	BBI 1 w/t RE	44.3	2.05
18	BBI 1 w/o RE	78.3	1.98
20	BBI 1 w/o RE	80.7	1.97

August 2

The TOP10 transformed with either pSG1154-*ComK* or pMLK83-*ComK* grew on the Amp. plates. These *ComK* constructs contained the AmyE integration sequences. Master plates of these colonies were made so that PCR could be performed tomorrow.

The BBI #5 with and without RE were cPCRed. The colonies selected were: BBI #5 w/t RE 1-5 and BBI #5 w/o RE 1&2. On the confirmation gel, there were no bands. More overnights of both BBI 5 with and without restriction enzymes were made.

August 3

The Homebrew of BBI #1 were redone for the third time...Also included were the BBI #5 constructs.

Colony	Plasmid	Yield (ng/uL)	A260/280
1	BBI #1 with RE	5	1.42
7	BBI #1 with RE	4.3	1.04
8	BBI #1 with RE	8.4	1.47
10	BBI #1 with RE	4.7	1.37
11	BBI #1 with RE	25.9	1.78
18	BBI #1 without RE	26.3	1.56
20	BBI #1 without RE	40.0	1.62
5	BBI #5 with RE	25.8	1.71
1	BBI #5 without RE	17.8	1.74

TOP10 was transformed with the two different chromoproteins used for our indicator system. The constructs are blue and pink chromoproteins with one of three promoters: BBa_K78003, Pveg, PsspB; BBa_K780003, Pveg, PsspB respectively. In total there were 6 different constructs.
Lastly, *B. subtilis* was streaked onto a LB plate and incubated overnight at 37 ℃.

August 4
B. subtilis was transformed with pSB3C5 plasmids containing BBI #1 or #5 with and without Restriction sites. *B. subtilis* was transformed following Dr. Wong's Protocol. Only one tube of SP1 media was inoculated with *B. subtilis* overnight culture as there were minimal number of colonies on the streak plate. The one tube of SP1 culture was split into 4 tubes each containing 4.5mL of SP2 media and 0.5mL of SP1 culture. 1 mL of SP2 culture from each tube was used for 2 transformations total. 20uL of DNA was used to transform the cells, with amount of DNA between 0.3-1 ug. 4 different constructs were used for two repeats each.

cPCR using TOP10 cells transformed with pMKL83-*ComK* was done. The cells were taken from the master plates made on August 2. Unfortunately only colony #15 was able to grow. 8 reactions total were run with a 9x master mix. Protocol used was the same from July 12.

The August 3 minipreps were redone as it was thought that the yields were too but this attempt didn't go that well either…

Colony	Plasmid	Yield (ng/ul)	A260/280
1	BBI #1 with RE	18.9	1.45
7	BBI #1 with RE	13.9	1.49
8	BBI #1 with RE	16.4	1.97
10	BBI #1 with RE	24.8	1.24
11	BBI #1 with RE	24.4	1.28
18	BBI #1 without RE	26.1	1.31
20	BBI #1 without RE	26.1	1.24
5	BBI #5 with RE	26.7	1.32
1	BBI #5 without RE	2.1	0.77

The extracted plasmids were then digested and run on a gel for confirmation. No DNA was seen in the gel unfortunately.
The TOP10 that were transformed with the chromoproteins did not grow and were then left to keep growing in the incubator at 37℃.

August 5
Samples of all four BBI constructs was sent to the sequencing for a more precise conformation relative to running PCR or restriction digest products on a gel.

August 8
The *Bacillus* cells that were transformed on August 4 were cPCRed. Eight samples were taken from each plate except for two plates that needed more time to grow (these were *Bacillus* transformed with

BBI 5 constructs). From the plates that took longer to grow, 4 and 2 samples were used. In total a 57x master mix was needed for the 56 samples run. The same protocol from July 12 was used.

August 9

Overnight cultures of the transformed *Bacillus* from August 4 were minipreped using the Omega-BioTek E.Z.N.A plasmid kit. The kit protocol was followed except that 10mL of culture was used for each of the 4 plasmids. This was to compensate the generally lower yields that we were getting from *Bacillus.* The Omega-BioTek E.Z.N.A plasmid kit was used as we wanted to send these plasmids down for sequencing and wanted the higher quality preps.

Plasmid	Yield (ng/ul)	A260/280
pSB3C5 BB1 #5 w/o RE	33.2	1.86
pSB3C5 BB1 #5 w/t RE	30.3	1.99
pSB3C5 BB1 #1	27.2	1.93
pSB3C5 BB1 #1	44.5	1.94

The six different chromoprotein constructs (blue or pink chromoproteins with either Pveg, PsspB, or BBa_K780003 promoters) were double digested with EcoRI and PstI. The constructs were then inserted into both pSB1C3 and pSB3C5 (both digested with EcoRI and PstI). The products were ligated and transformed into TOP10. The transformed cells were incubated at 37°C overnight.

August 10

Prepared eight samples for sequencing. There were two samples for each of the plasmids, forward and reverse. 150ng of template were used for each of the samples in order to be within the 50-100ng range/1 kb. 1uL of primer was used for each sample, either prefix-forward or suffix reverse depending on whether the sample was forward or reverse. The remaining volume was filled to 12uL with water.

The transformed TOP10 from yesterday grew with more growth from the cells transformed with pSB1C3-chromoproteins than the equivalent pSB3C5 (the low copy number plasmid). The transformed cells were then used to make master plates for the 1C3 plasmid but not 3C5 due to low growth. The 3C5 transformed cells were left to grow in the incubator for another 24 hours.

August 11

After the extra incubation period, the pSB3C5 transformed TOP10 cells had grown to roughly the same level as the pSB1C3 transformed cells. Thus, a master plate was made for the 3C5 transformed cells and incubated overnight at 37°C.

The 1C3 transformed cells were then used for cPCRing of all the chromoprotein constructs. On the confirmation gel there were sadly no bands apart from the ladder.

August 12

The cPCR from yesterday was repeated but using colonies from 3C5-Chromoprotein transformed TOP10. On the verification gel there were no bands for any of the blue transformants but the pink transformants had consistent bands at 1.1kb.

August 15

The protocol to sporulate *Bacillus* was started. Overnights were made using *Bacillus* from the untransformed master plate.

The cPCR from August 11 and 12 was repeated. The products were run again on a gel to confirm PCR.

pSB1C3 transformed TOP10		pSB3C5 transformed TOP10	
Pink Chromoprotein	Blue Chromoprotein	Pink Chromoprotein	Blue Chromoprotein
Consistent band at 800 base pairs for all three types of promoters	Consistent band at 800 base pairs for all three types of promoters	Consistent band at 800 base pairs for all three types of promoters	Consistent band at 800 base pairs for all three types of promoters

August 16

pSB3C5 plasmids minipreped from the overnight cultures from August 15. Because the cultures grew less than expected, the tubes were left in the incubator till later in the afternoon. The minipreps were done using the Omega Biotek E.Z.N.A plasmid mini kit and followed the protocol+Rachelle's notes. 10ml of culture was used to offset the generally low yields from *Bacillus* and the low copy number plasmid. The yields that were obtained were low, ranging from 16.0-28.9ng/ul, with a A260/280 values of 1.88-2.19.

The plasmids containing the chromoproteins were minipred with the homebrew kit, using the overnights made from the successful cPCRs from August 14.

Plasmid	Blue or Pink?	Promoter+Colony#	Yield (ng/ul)	A260/280
pSB1C3	Blue	Pveg 6	51.9	1.86
pSB1C3	Blue	PsspB 5	63.5	1.98
pSB1C3	Blue	PsspB 6	50.9	1.88
pSB1C3	Blue	BBa_K780003 5	77.5	1.92
pSB1C3	Blue	BBa_K780003 6	24.9	1.51
pSB1C3	Pink	Pveg 5	43.7	1.95
pSB1C3	Pink	Pveg 6	172.3	1.95
pSB1C3	Pink	PsspB 5	63.6	1.94
pSB1C3	Pink	PsspB 6	29.1	1.72
pSB1C3	Pink	BBa_K780003 5	44.6	1.96
pSB1C3	Pink	BBa_K780003 6	293.5	1.91
pSB3C5	Blue	BBa_K780003 3	73.5	1.96
pSB3C5	Blue	BBa_K780003 4	41.9	1.89

pSB3C5	Blue	PsspB 3	60.1	1.99
pSB3C5	Blue	PsspB 4	67.1	1.94
pSB3C5	Blue	Pveg 3	261.6	2.00
pSB3C5	Blue	Pveg 4	27.5	1.94
pSB3C5	Pink	Pveg 1	41.3	1.95
pSB3C5	Pink	Pveg 2	19.4	1.97
pSB3C5	Pink	Psspb 1	20.9	1.66
pSB3C5	Pink	BBa_K780003 2	33.5	1.89

Inserts of *ComK* with *AmyE* homology regions inside the prefix and suffix (new) and with *AmyE* homology regions outside the prefix and suffix (old) were digested with EcoRI and SpeI following the protocol on page 5 of Rachelle's lab book. Plasmids pSB1C3-RFP, pSB3C5-I52001 and pSG1154 were also digested with EcoRI and SpeI following the same protocol. The old *ComK* insert will be ligated into pSG1154 for future chromosomal integration in *B. subtilis* as well as pSB1C3 for amplification, storage and registry submission. The new *ComK* insert will be ligated into pSB3C5 to mimic the effects of multiple sites of chromosomal integration (as it is a low copy number plasmid, as well as pSB1C3 for amplification, storage and registry submission.

The pSB1C3, pSB3C5 and pSG1154 vectors were treated with Antarctic phosphatase as per the protocol on page 5 of Rachelle's lab book to inhibit the plasmids from self-ligation. The following was added to each tube of digested vector (4 tubes total, 1 pSB3c5, 1 pSG1154 and 2 pSB1C3):

5 uL 10X Antarctic phosphatase buffer
9 uL ddH2O
1 uL Antarctic phosphatase

The following combination of *ComK* insert to vector was performed following the ligation protocol

Old *ComK* (without *AmyE*) into pSB1C3
Old *ComK* (without *AmyE*) into pSG1154
New *ComK* (with *AmyE*) into pSB1C3
New *ComK* (with *AmyE*) into pSB3C5

The products were stored at -20°C.

Since we thought our competent cell preparations weren't very good, we did one quality control transformation with a randomly selected aliquot. One aliquot of competent TOP10 was transformed with 10ul pSB1C3-RFP at 40.3ng/ul as per the standard transformation protocol for *E. coli*. The transformants were plated on Chlor plates and incubated overnight at 37℃.

Note: there was initially no growth but after a replating and another day of incubation, RFP expressing cells were observed on the plates but at a very low transformation efficiency

August 17

Samples for sequencing we prepared using 160-200ng of template DNA. The template DNA are all four of the BBI constructs. 1 ul of either forward or reverse primer was used in each tube and the remaining volume to 12ul was filled with ddH_2O. In total there were eight reactions, four forward and backwards.

More work was done on sporulating cells. The overnight cultures were diluted 1:200 in the 2xSG media and incubated at 37°C and 200rpm. The culture was checked for spores approximately every 24 hours using a gram stain. The protocol was the same one used by the 2014 Calgary iGEM team. The protocol can be found on Open Wet Ware.

Minipreps of the chromoprotein plasmids were redone but only for the ones that had a low yield. The new miniprep did not improve on the previous low yields from August 16.
However the well preped samples from August 16 were prepared and sent down for sequencing.

August 18
To check for the presence of spores in culture, a gram stain was performed. Spores will not stain and remain colourless as the spores are impermeable to any of the stains while the unsporulated *Bacillus* will stain purple (gram positive). Under the microscope there were very few spores present ~<10%.

4 aliquots of TOP10 cells were transformed with the ligated products from August 16 following the transformation protocol. 10ul of ligation products were used in the following transformations:

pSB1c3 - old *ComK*	plated on Chlor	
pSG1154 - old *ComK*	plated on Amp	
pSB1c3 - new *ComK*	plated on Chlor	
pSB3c5 - new *ComK*	plated on Chlor	
pSB1c3 - RFP	plated on Chlor	positive control

August 19
The second gram stain of the developing spores revealed greater sporulation than August 18 but there was not a clear majority of spores relative to vegetative cells.

The *Bacillus* that was streaked the previous night was then transformed with the plasmids (pSB1C3 and pSB3C5) containing the chromoproteins and their respective promoters.
The TOP10 transformed on August 18 failed to grow on any of the plates. Those plates were left in the incubator while fresh plates were spread with transformed cells from August 18. And because we think there are issues with the cells that were made competent, an untransformed aliquot of cells from the glycerol stocks were streaked and incubated at 37°C.

August 20
Still no growth on either set of plates...we think it's the competent cells, they were made from an old batch of untransformed *E. coli* TOP10 and also took a really long time to grow to an OD600 of 0.4 during competency protocol.

Gram staining of the *Bacillus culture* revealed that the vast majority of the cells had sporulated. This was seen under the microscope as colourless coccoid objects. Spores (10mL) were removed from the original culture and spun down for 2 minutes at 14k rpm. The spores could be differentiated from vegetative cells by the dark brown colour of the spores. Isolated spores were stored in fresh ddH_2O at -20°C.

> **Note**: This was all that was done with the spores as we had to focus on the expression of BBI over an sporulation experiments. We figured that we could use the 2014 UCalgary Team data if necessary.

August 21
New, fresh cells were made competent as per the protocol on page 2 of Rachelle's lab book. During resuspension (step 4) and aliquotting (step 6), the procedure was performed in the cold room at 4°C. Cold pipette tips and aliquot tubes were used throughout the protocol. Step 6 was performed (samples were placed on ice for 30 min) before storing aliquots at -80°C.

August 22
The competent cells that were made yesterday were tested by transforming 3 aliquots of freshly-made competence cells with the following samples of DNA (following the normal transformation protocol.

Tube 1: 7.4 uL of pSB1c3-RFP (120 ng/uL)
Tube 2: 1 uL of pSB1c3-RFP (0.2 ng/uL)
Tube 3: nothing (negative control)

After adding DNA, the aliquots were left to sit on ice for 1 hour (instead of 30 minutes) to allow for DNA to enter each cell.

After transforming, 100 uL of sample was plated on Chlor plates and left to incubate at 37°C O/N.

The *Bacillus* that was transformed yesterday grew on the LB-agar plates.

August 23
Growth was observed on both RFP transformed plates and none on the negative control plate (**our cells are competent!**)

4 aliquots of competent TOP10 cells were transformed with the following ligated *ComK* products as per the normal transformation protocol.

Tube 1: pSC1c3-*ComK* without homology (11.25 uL)	plated on Chlor
Tube 2: pSG1154-*ComK* without homology (10.5 uL)	plated on Amp
Tube 3: pSB1c3-*ComK* with *AmyE* homology (10.4 uL)	plated on Chlor
Tube 4: pSB3c5-*ComK* with *AmyE* homology (11.0 uL)	plated on Chlor

After adding DNA to the competent cell aliquots, the cells were left on ice for 1 hour instead of 30 minutes. 100 uL of each samples was plated on the appropriate plate instead of 50 uL. Cells were left to incubate at 37°C O/N.

pSB1c3-RFP was prepped from the O/N prepared yesterday by following the homebrew protocol which gave the following yields.

Tube 1 of pSB1c3-RFP:	60.9 ng/uL	A260/280: 2.11
Tube 2 of pSB1c3-RFP:	56.3 ng/uL	A260/280: 1.54
Tube 3 of pSB1c3-RFP:	58.9 ng/uL	A260/280: 1.96
Tube 4 of pSB1c3-RFP:	58.2 ng/uL	A260/280: 2.35

The prepped pSB1c3-RFP plasmids were singly and doubly digested following Rai's protocol.

Plasmid	Single Digest	Double Digest
Tube 1: pSB1c3-RFP (60.9 ng/uL	EcoRI	EcoRI + PstI

Tube 2: pSB1c3-RFP (56.3 ng/uL)	EcoRI	EcoRI + PstI
Tube 3 of pSB1c3-RFP	EcoRI	EcoRI + PstI
Tube 4: pSB1c3-RFP (58.2 ng/uL)	EcoRI	EcoRI + PstI

The 8 digests were run on a 1% agarose gel using an Amresco 1 Kb DNA ladder

Lane	Contents	Expected Size (bp)	Observed Size (bp)
1	LAdder		
2	pSB1c3-RFP #1, single digest	3100	3100
3	pSB1c3-RFP #1, double digest	1000, 2100	Nothing
4	pSB1c3-RFP #2, single digest	3100	3100
5	pSB1c3-RFP #2, double digest	1000, 2100	Nothing
6	pSB1c3-RFP #3, single digest	3100	3100, 5000
7	pSB1c3-RFP #3, double digest	1000, 2100	1000, 2100, 2500
8	pSB1c3-RFP #4, single digest	3100	3100, 5000
9	pSB1c3-RFP #4, double digest	1000, 2100	1000, 2100, 2500

Based on the gel, we have to redo the minpreps. There was obviously something wrong as the 5000bp was 2000bp larger than any of plasmids miniprepped!

August 25

Following the *Bacillus* transformation protocol from Dr. Wong, *Bacillus* was transformed with the 3 different promoter+chromoprotein constructs in both pSB1C3 and 3C5 vectors. The transformed cells were streaked onto LB-Chlorplates and incubated overnight at 37°C.

The *ComK* inserts with and without *AmyE* homology were PCR amplified using the Prefix-F and Suffix-R primers and the PCR protocol found on page 6 of Rachelle's lab book. The products were run on a 1 agarose gel with a 1 KB PLus DNA Ladder.

Lane	Contents	Expected Size (bp)	Observed Size (bp)
1	amplified *ComK* without homology		
2	amplified *ComK* with *AmyE* homology	970	970

3	DNA ladder	1200	1200

The inserts were successfully amplified, hallelujah! They were nanodropped to give the following yields:

Amplified *ComK* without homology 85 ng/uL
Amplified *ComK* with *AmyE* homology 60 ng/uL

4 vector tubes and 3 insert tubes were digested and treated with fast Antarctic Phosphatase following standard digestion protocol on page 5 of Rachelle's lab book:

pSB1c3 tube #1
10 uL pSB1c3 (50 ng/uL)
3 uL 10X Cutsmart buffer
1 uL XbaI
1 uL SpeI
1 uL Fast Ap
In 30 uL total with ddH2O

pSB1c3 tube #2
10 uL pSB1c3 (50 ng/uL)
3 uL 10X Cutsmart buffer
1 uL XbaI
1 uL SpeI
1 uL Fast Ap
in 30 uL total with ddH2O

pSG1154 tube
5 uL pSG1154 (200 ng/uL)
3 uL 10X Cutsmart
1 uL EcoRI
1 uL SpeI
1 uL Fast Ap
In 30 uL total with ddH2O

pAB3c5 tube
1 uL pSB3c5 (545 ng/uL)
3 uL 10X Cutsmart
1 uL XbaI
1 uL SpeI
1 uL Fast Ap
in 30 uL total with ddH2O

ComK without homology tube #1
10 uL *ComK* without homology (85 ng/uL)
2 uL 10X Cutsmart
1 uL XbaI
1 uL SpeI
In 20 uL with ddH2O

ComK without homology tube #2
10 uL *ComK* without homology (85 ng/uL)
2 uL 10X Cutsmart
1 uL EcoRI
1 uL SpeI
in 20 uL with ddH2O

ComK with *AmyE* Homology tube
13 uL *ComK* with *AmyE* Homology (60 ng/uL)
2 uL 10X Cutsmart
1 uL XbaI
1 uL SpeI
In 20 uL with ddH2O

All 7 tubes were left to digest at 37°C for 2 h before the restriction enzymes were heatkilled at 80°C for 20 minutes. Products were stored at 4°C O/N

August 26

The plasmid were miniprepped from the overnight cultures of transformed *Bacillus* from August 25. The plasmids were then sent to be sequenced. Three constructs were sequenced: Pveg+Blue chromoprotein in pSB3C5, PsspB+Pink in pSB1C3, and BBa_K780003+Pink in 3C5.

ComK was ligated into 3 different vectors in the following procedure. 4 ligation tubes containing the following insert and vector combinations were ligated for 6 hours at room temperature:

1. pSB1c3-*ComK* without homology
3.5 uL pSB1c3 digested with XbaI + SpeI (16.7 ng/uL)
7 uL *ComK* without homology digested with XbaI + SpeI (42.5 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4 DNA ligase
In 20 uL total ddH2O

2. pSG1154-*ComK* without homology
1.5 uL pSG1154 digested with EcoRI + SpeI (16.7 ng/uL)
10 uL *ComK* without homology digested with EcoRI + SpeI (42.5 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4 DNA ligase
In 20 uL total ddH2O

3. pSB1c3-*ComK* with *AmyE* homology
3.5 uL pSB1c3 digested with XbaI + SpeI (16.7 ng/uL)
7 uL *ComK* with *AmyE* homology digested with XbaI + SpeI (39 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4DNA ligase
In 20 uL total ddH2O

4. pSB3c5-*ComK* with *AmyE* homology
3 uL pSB3c5 digested with XbaI + SpeI (31.3 ng/uL)
4.5 uL *ComK* with *AmyE* homology digested with XbaI + SpeI (39 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4 DNA ligase
In 20 uL total ddH2O

The ligated *ComK* products from above (tubes 1-4) and a pSB1c3-YFP plasmid from the 2016 iGEM registry kit (plate 4, well 2M) were used to transform 5 aliquots of competent *E. coli* TOP10 cells following the protocol on page 2 of Rachelle's lab book. 100 uL of each sample was plated on the appropriate plate (Chlor for pSB1c3/3c5 plasmids, Amp for pSG1154 plasmid) and left to incubate O/N at 37°C.

August 29
Sequencing results came back for the chromoprotein constructs and it was discovered that the sequences aligned perfectly to RFP. The sequences for when the chromoproteins were transformed into *E. coli* were checked as well and we discovered that, they too, were RFP.

September 6
An overnight culture (5mL) of *Bacillus* transformed with BBI 1 was started and left to incubate throughout the day at 37°C and 200rpm. While the culture was incubating, 1L of plain LB broth was prepared and autoclaved in a 2L Erlenmeyer flask. At 5pm, the media was inoculated with the 5mL of *Bacillus* culture. The 1L culture was left to grow overnight at 37°C and 200 rpm.

September 7
800mL of overnight culture was taken out of the 2L flask and transferred to several 50mL falcon tubes. Then through a series of spin downs and suspensions, the *Bacillus* was concentrated into one mega pellet in a single falcon tube. The mega pellet was then resuspended in 10mL of ddH_2O.

The resuspended cells were then sonicated in order to rupture the cell membranes. This was necessary in order to measure the amount of BBI produced by the cells but not secreted. The sonicator was set to pulse for 30 seconds followed by 30 second pause. This cycle repeated 5 times. The amplitude of the pulse was 30% of maximum power. We were given a demonstration in how to operate the sonicator from Ms. Linda Lee who was in charge of it. The broken up cells were then aliquoted into 2mL tubes and spun down for 10 minutes at 14k rpm. The samples were stored at -20℃ overnight.

September 8
Samples of the cell lysate and media were diluted 1:10, 1:100, and 1:1000 and delivered to Dan for analysis with mass spectrometry. There were no results unfortunately.

September 9-October 14
Attempting to clone our assorted parts into the BioBrick backbone (pSB1C3). This has had limited success. We realized that we never had BBI in any plasmid and even worse, all of our stocks had been degraded. Forcing us to get more from IDT. At the current point in time the following parts have been parts have been cloned into the BioBrick backbone: *E. coli* BBIGFP, *B. sub* BBI#1GFP, and *B. sub* BBI#5GFP.

October 15-18
Cloned all submitted parts into the pSB1C3 plasmid. All were sequenced confirmed and can be accessed from the iGEM registry. These are BBa_K2008001 (http://parts.igem.org/Part:BBa_K2008001), BBa_K2008002(http://parts.igem.org/Part:BBa_K2008002), BBa_K2008003(http://parts.igem.org/Part:BBa_K2008003), BBa_K2008004(http://parts.igem.org/Part:BBa_K2008004), BBa_K2008005(http://parts.igem.org/Part:BBa_K2008005), BBa_K2008006(http://parts.igem.org/Part:BBa_K2008006), BBa_K2008007(http://parts.igem.org/Part:BBa_K2008007), BBa_K2008008(http://parts.igem.org/Part:BBa_K2008008).

The the parts were diluted to 25ng/uL and 10ul of each sample was placed in their respective well of the shipping plate. The plate was then covered with the lid and allowed to dry overnight in the bio-safety cabinet (BSC) as the BSC has a continuous airflow and would be more sterile than the fumehood.

October 19
The dried samples were sent to Boston via Fedex!